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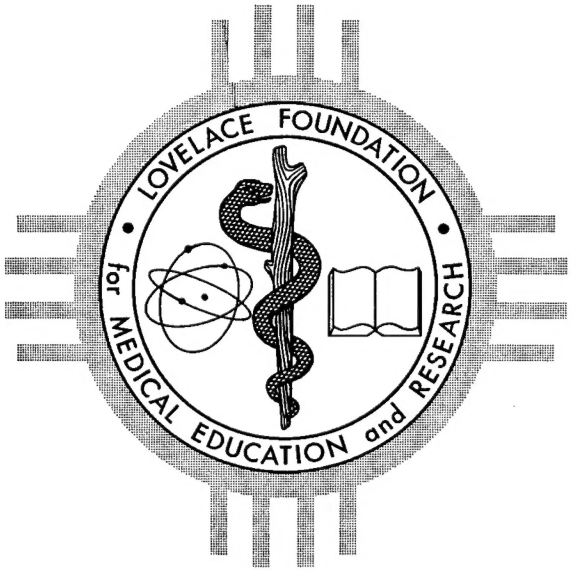

Albuquerque, New Mexico

ULTRAMICRO METHODS IN BIOCHEMISTRY

V. A COMPARISON OF THE ULTRAMICRO DETERMINATION OF PHOSPHORUS USING N-PHENYL-p-PHENYLENEDIAMINE OR 1-AMINO-2-NAPHTHOL-4-SULFONIC ACID

VI. THE DETERMINATION OF SERUM ALKALINE PHOSPHATASE

by

E. VAN STEWART
AND
BERNARD B. LONGWELL

December 1964

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VI. THE DETERMINATION OF SERUM ALKALINE PHOSPHATASE

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E. Van Stewart and Bernard B. Longwell

with the technical assistance of Agnes Wood and Tom Tenney

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ABSTRACT

An ultramicro method has been developed for the determination of serum inorganic phosphorus which uses 1-amino-2-naphthol-4-sulfonic acid as the reducing agent. This procedure is compared to an ultramicro method which applies the reaction induced by N-phenyl-p-phenylene-diamine. The acceptable wavelengths for the measurement of absorbance in both methods of analyses have been investigated and are discussed. The two methods are comparable in reproducibility and give results which justify the use of either one for the determination of phosphorus.

The method using 1-amino-2-naphthol-4-sulfonic acid has been applied to the determination of serum alkaline phosphatase with a substrate consisting of β-glycerophosphate in a barbiturate buffer at pH 9.3. The ultramicro method is compared to the macro method from which it was derived. The comparisons of results with the two procedures are within acceptable limits to justify recommendation of the ultramicro method for the determination of alkaline phosphatase.

ACKNOWLEDGMENTS

The authors wish to acknowledge with thanks the cooperation of Dr. Clayton S. White, Mr. Robert Smith and the personnel of the Department of Medical Illustration, and the secretarial assistance of Mrs. Beth Bowen, Mrs. Eleanor Scotten, and Mrs. Nancy Nichols.

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INTRODUCTION

The method of Fiske and Subbarow (1) is widely used in clinical chemistry for the determination of phosphorus. Micro modifications of this method, which uses 1-amino-2-naphthol-4-sulfonic acid (ANSA) as the reducing agent, have been fraught with difficulties and it has even been suggested that ANSA could not be used on the ultramicro scale (2, 3). The following report describes an ultramicro procedure using ANSA as the reducing agent. The method is a modification of the method of Kaser and Baker (4). Results obtained with this method are compared to results obtained with the procedure of Dryer, Tammes, and Routh (2) adapted for ultramicro analysis (3). The latter method uses N-phenyl-p-phenylenediamine (NPPD) as the reducing agent.

METHOD

A. Equipment

The main components of the Sanz-Spinco ultramicro clinical chemistry system (5) were used with the following exceptions:

1. Pipettes. All pipettes larger than 20μl capacity were made and calibrated in this laboratory.

2. Colorimeter. Both the Coleman Junior spectrophotometer equipped with an ultramicro cell and the Spinco spectrocolorimeter which had been modified to accept the Coleman ultramicro cell were used.

3. Mixer. A Vortex Junior Mixer (Scientific Industries, Queens Village, New York) was substituted for the Spinco micromixer.

B. Reagents for Using Aminonaphtholsulfonic Acid (ANSA)

1. Phosphorus standard (stock) (40 mg/100 ml). Dissolve 0.176 gm of dried potassium dihydrogen phosphate (KH_2PO_4) in distilled water and dilute to 100 ml. The stability is increased if this standard is kept in the refrigerator.

2. Dilute standard (4 mg/100 ml). Dilute 1.0 ml of the stock standard to 10 ml with water.

3. Trichloroacetic acid (10%). Dissolve 10 gm of trichloroacetic acid in distilled water and make up to 100 ml.

4. Molybdic acid. Dissolve 25 gm of reagent grade ammonium molybdate in 500 ml of distilled water. Add 300 ml of 10 percent sulfuric acid and dilute to one liter with water.

5. Aminonaphtholsulfonic acid (ANSA). Dissolve 14.6 gm of sodium hydrogen sulfite ($NaHSO_3$) and 0.5 gm of sodium sulfite (Na_2SO_3) in distilled water and make up to 100 ml. Add 0.1 gm of powdered 1-amino-2-naphthol-4-sulfonic acid and shake until it has dissolved. Store in a brown bottle in the refrigerator. If the solution is colored, filter through activated charcoal.

C. Procedure

1. To a 400 μl polyethylene test tube add 10 μl of blank (distilled water), standard, or serum followed by 150 μl of 10 percent trichloroacetic acid.

2. Mix thoroughly and let stand for two minutes, then centrifuge for one and one-half minutes.

3. Transfer a 100 μl aliquot to a clean micro test tube.

4. Add 20 μl of molybdic acid and mix. Then add 20 μl of ANSA and mix thoroughly.

5. Let stand for 15 minutes and read absorbance against water at 650 mμ with the Spinco spectrocolorimeter, or 680 mμ with the Coleman Junior spectrophotometer, in a micro cuvette.

D. Calculation

$$\frac{\text{Absorbance (A) of unknown - A of blank}}{\text{A of standard - A of blank}} \times 4 = \text{mg phosphorus/100 ml}$$

E. Method Using N-Phenyl-p-Phenylenediamine (NPPD)

The method of Dryer, Tammes, and Routh (2) as modified for ultramicro analysis by Knights, MacDonald, and Ploompuu (3) was used as described, with the exception of one experiment in which a 20μl sample was substituted for the 25μl sample originally suggested.

RESULTS

A. Choice of Wavelength

Some instruments do not provide a selection of wavelength greater than 650 mμ. Because of this limitation, the feasibility of phosphorus analyses with absorbance measurements at wavelengths other than the point of maximum absorptivity was investigated. Absorbances were measured at 650, 680, 700, and 745 mμ with standards equivalent to 2 to 10 mg phosphorus per 100 ml. The readings were made with a Beckman DU spectrophotometer modified for ultramicro analysis and equipped with the Gilford[1] Instrument multiple sample absorbance recorder.

Curves 1, 2, and 3 (Figure 1) show the results obtained with the ANSA method as described above when the absorbance measurements were made at 700, 680, and 650 mμ, respectively. Curves 4, 5, and 6 record measurements made at the same wavelengths, but with the method modified to use a 20μl sample with a final dilution of the color reaction to 320μl, the same volume used in the NPPD method. Figure 2 presents standardization data for the NPPD method with a 20μl rather than the 25μl sample originally prescribed. The absorbance measurements were made at 700, 680, and 650 mμ for Curves 1, 2, and 3, respectively.

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[1]Gilford Instrument Laboratories, Inc., Oberlin, Ohio.

The graph of the results at 700 and 745 mμ for both methods was so nearly identical that only the values obtained at 700 mμ are plotted. The comparative values are shown below.

Phosphorus concentration mg per 100 ml	ANSA Method		NPPD Method	
	700 mμ	745 mμ	700 mμ	745 mμ
2	0.086	0.083	0.063	0.063
4	0.175	0.172	0.125	0.126
6	0.265	0.264	0.188	0.190
10	0.441	0.439	0.322	0.324

It is evident that wavelengths from 650 to 745 mμ may be used with either of these methods to give results that are acceptable for the analyses of serum phosphorus. The differences in absorbances are explained by differences in sample size, final color reaction volumes and color intensity developed.

B. Standard Curve

The results recorded in Figure 3 show the absorbance values for phosphorus standards of varying concentrations. Curve 1 shows the results with the ANSA method as described herein under Procedure (10μl sample, final color reaction volume 140μl). The absorbance measurements were made at 680 mμ with the Coleman Junior spectrophotometer equipped with an ultramicro cuvette. Curve 2 records results with the ANSA method modified by using a 20μl sample and making a final dilution to 320μl to correspond to the final volume of the color reaction of the NPPD method. Curve 3 shows the results of the NPPD method as described by Knights, MacDonald, and Ploompuu (3) with their designated 25μl sample. The readings for Curve 2 were made at 680 mμ and for Curve 3 at 700 mμ. All absorbance measurements were made with the Coleman Junior spectrophotometer.

Absorbance is linear with concentration by both methods over the ranges recorded in Figure 2, up to 10 mg phosphorus per 100 ml. The close agreement of the results obtained with the ANSA method, modified

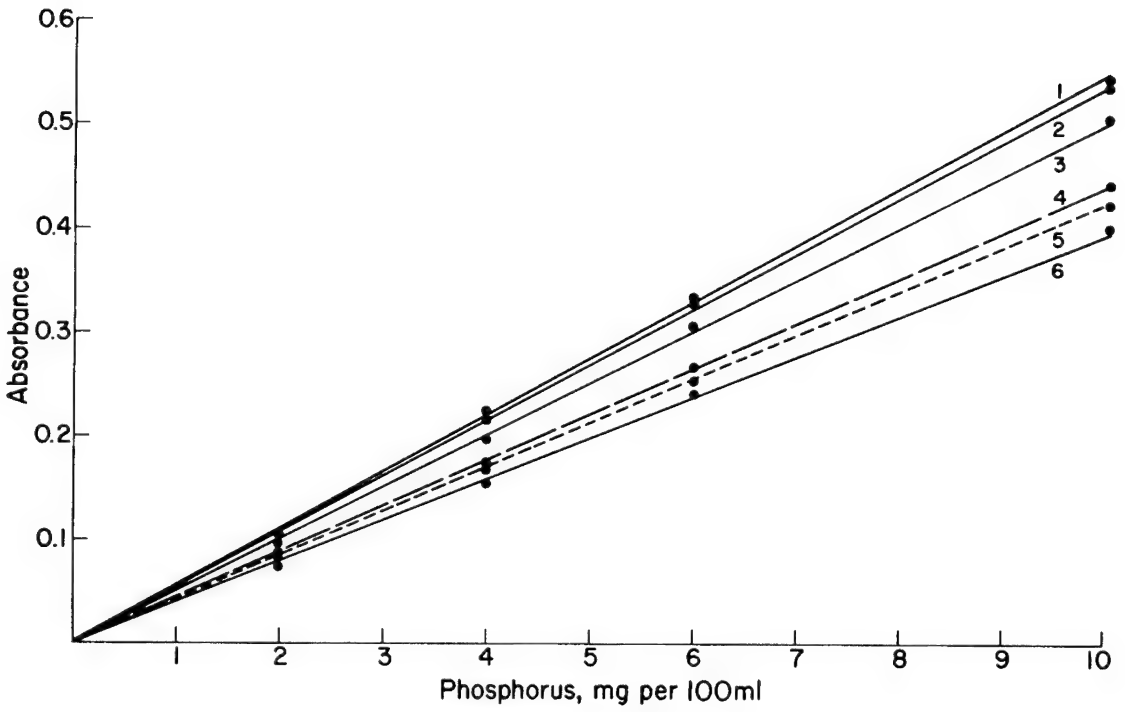

Fig. 1. Absorbance with ultramicro phosphorus method using 1-amino-2-naphthol-4-sulfonic acid as the reducing agent. Curves 1, 2, and 3 show absorbance values at 700, 680, and 650 mμ, respectively, with the method as described in the text. Curves 4, 5, and 6 show absorbance values at 700, 680, and 650 mμ obtained with the method modified to use a 20μl sample followed by dilution of the color reaction to 320μl.

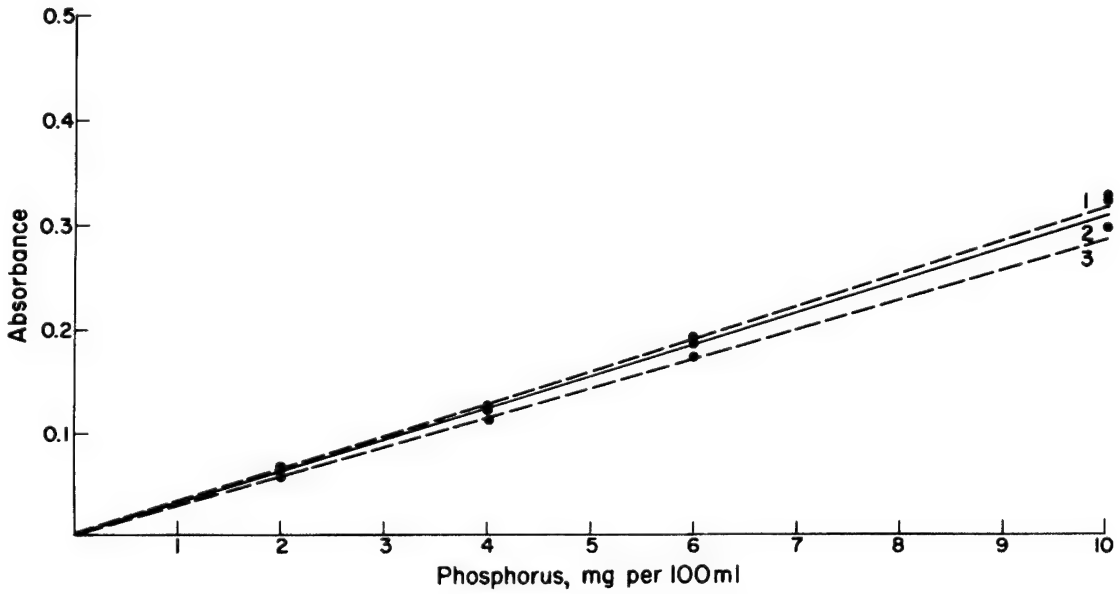

Fig. 2. Absorbance values obtained with the ultramicro method for phosphorus analysis which uses N-phenyl-p-phenylenediamine as the reducing agent (3). The method was modified to use a 20μl sample. Curves 1, 2, and 3 were obtained at 700, 680, and 650 mμ, respectively.

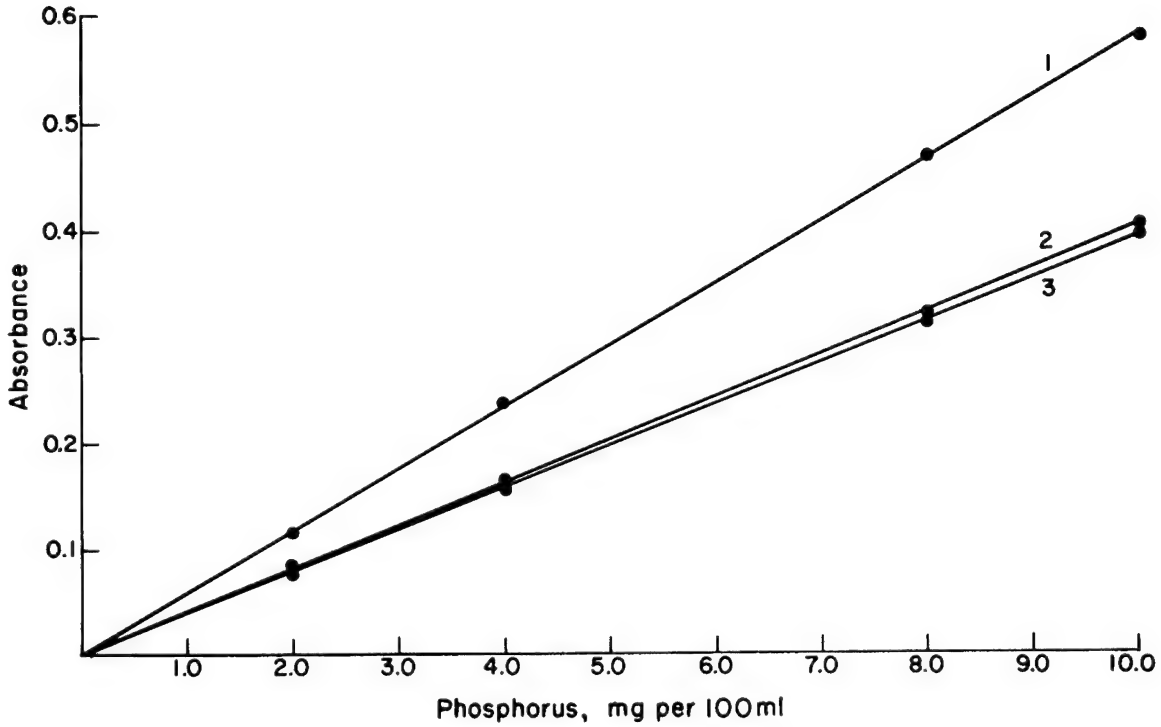

Fig. 3. Standardization curves for two ultramicro methods for phosphorus determination. Curve 1, the method using 1-amino-2-naphthol-4-sulfonic acid described in the text. Curve 2, the aminonaphtholsulfonic acid method modified to use 20μl of sample followed by final color reaction dilution to 320μl. Curve 3, method using N-phenyl-p-phenylenediamine as the reducing agent (3).

to compare approximately in sample size and exactly in final dilution with the NPPD method, indicates that there is little to choose between the two reducing agents applied to the ultramicro determination of phosphorus.

C. Standard Deviations

Standard deviations were determined for both procedures by doing thirty analyses on the same samples. A different serum sample was used for the determination of the standard deviation for each of the methods. The modified Fiske and Subbarow method gave a standard deviation of $\pm$ 0.05 on a serum with a mean phosphorus value of 3.2 mg per 100 ml and a standard deviation of $\pm$ 0.12 was obtained on a serum with a mean phosphorus value of 4.46 mg per 100 ml of serum by the NPPD procedure.

D. Recovery of Phosphorus

Dried crystalline potassium dihydrogen phosphate was added to an aliquot of a fresh serum sample which was then mixed thoroughly until the crystals were dissolved. The phosphorus was determined on an aliquot without added phosphorus and on an aliquot with added phosphorus by both methods. By calculation, 3.7 mg of phosphorus was added per 100 ml of serum. A recovery of 3.8 mg per 100 ml was obtained with the NPPD method and 3.76 mg per 100 ml with the ANSA procedure, recoveries of 103 and 102 percent, respectively.

E. Comparison of Results

Thirty serum samples were analyzed for phosphorus by both methods. A comparison of the results is shown in Table 1. Good agreement was obtained between the methods on the majority of samples. The exceptions were those sera which were lipemic. In the lipemic samples, no definite trend was apparent which indicated that one method was giving consistently higher results than the other. In two of the samples, the value obtained by the ANSA method was higher, while in three of the samples, the NPPD method gave the higher values. No explanation

TABLE 1

COMPARISON OF THE ANSA AND NPPD METHODS ON SERUM SAMPLES

Sample	mg of Phosphorus per 100 ml of serum	
	ANSA Method	NPPD Method
1	3.9	4.0
2	3.1	3.3
3	3.9	4.1
4	4.4	4.2
5	4.0	3.9
6	4.4	4.9 *
7	3.9	4.4
8	3.3	3.1
9	3.9	3.9
10	4.4	4.4
11	3.9	4.0
12	5.8	6.5 *
13	4.7	4.7
14	3.6	3.4
15	3.6	3.3
16	3.6	3.3
17	3.8	3.2 *
18	3.7	3.8
19	4.1	3.9
20	3.4	3.4
21	4.1	4.2
22	3.4	3.8
23	3.9	3.8
24	4.2	3.7 *
25	4.3	4.8 *
26	3.5	3.6
27	4.3	4.1
28	4.0	3.8
29	12.5	12.2
30	5.2	5.0

* Lipemic serum.

of these results, or indication of which, if either, method is more accurate in the presence of lipemia, can be advanced.

DISCUSSION

The results obtained by both the ultramicro NPPD and the ANSA methods are quite accurate and reproducible. Neither method appeared to have enough advantages over the other that it could be recommended as the method of choice.

The longest wavelength that can be used on the Spinco spectrocolorimeter is 650 mμ. Dryer et al. (2) showed that absorbance at 770 mμ is higher than at 650 mμ. The question, therefore, arose concerning whether wavelengths other than the maximum previously investigated could be used justifiably. This question seems to be answered by the data presented in Figures 1 and 2 up to a wavelength of 745 mμ. There is not sufficient difference between the absorbance values at the four wavelengths investigated to invalidate the use of any one of them for the determination of phosphorus. A determination of the ratios of absorbance at 770 mμ and 650 mμ for the two methods, using the Beckman DK-1A spectrophotometer, gave 1.19 for the NPPD method and 1.13 for the ANSA method.

During the transfer of the supernatant solution after precipitation and centrifugation of the proteins of some serum samples, a small portion of the precipitate was transferred in both methods. Longer periods of centrifugation did not seem to allow a cleaner transfer because a portion of the precipitate still remained on the surface around the edges of the liquid. It was found, however, that the interference from this precipitate could be eliminated by centrifuging the sample for one minute after the color had been developed for 15 minutes.

SUMMARY

A method for the ultramicro determination of phosphorus utilizing aminonaphtholsulfonic acid as the reducing agent has been evaluated and is compared to a method which employs N-phenyl-p-phenylenediamine. Either method is satisfactory for this determination.

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V. A COMPARISON OF THE ULTRAMICRO DETERMINATION OF PHOSPHORUS USING N-PHENYL-p-PHENYLENEDIAMINE OR 1-AMINO-2-NAPHTHOL-4-SULFONIC ACID

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ULTRAMICRO METHODS IN BIOCHEMISTRY
VI. THE DETERMINATION OF SERUM ALKALINE PHOSPHATASE

INTRODUCTION

Since the introduction of the Bodansky method for the determination of serum alkaline phosphatase (1), modifications of this procedure (2, 3) have been described, and several other approaches to the determination of this enzyme have been proposed (4, 5, 6). Procedures for ultramicro or micro analyses of alkaline phosphatase have been described by Bessey, Lowry, and Brock (4) and by O'Brien (7), Knights, MacDonald and Ploompuu (8), and Meites (9).

The present report describes the ultramicro determination of alkaline phosphatase by a modification of the method of Kaser and Baker (3) in which the phosphorus determination is accomplished as previously described (10).

METHOD

A. Reagents

1. The stock phosphorus standard, the dilute standard, the molybdic acid reagent and the aminonaphtholsulfonic acid reagent are identical to the same solutions described in the procedure for the determination of inorganic phosphorus (10).

2. Trichloroacetic acid (30%). Dissolve 30 gm of reagent grade trichloroacetic acid in distilled water and dilute to 100 ml.

3. Substrate. Dissolve 1.25 gm of sodium-β-glycerophosphate ($C_3H_9PO_6 \cdot 5H_2O$) and 1.06 gm of sodium diethyl barbiturate in approximately 100 ml of distilled water. Make up to 250 ml. Adjust the pH to 9.8 at 25°C, using either sodium hydroxide or hydrochloric acid. Divide the substrate into 10-20 ml portions and freeze. Keep frozen until used.

B. Procedure

1. To a 400 µl polyethylene test tube add 150 µl of substrate and 10 µl of serum. Mix thoroughly and incubate at 37°C for one hour.

While the phosphatase tubes (Procedure, Paragraph 1) are incubating, prepare the blanks, standard, and serum samples for the determination of inorganic phosphorus as follows:

2. To a 400 µl polyethylene test tube add 150 µl of substrate and 10 µl of blank (distilled water), standard, or serum. Immediately after the addition of a sample to each tube, add 50 µl of 30 percent trichloroacetic acid and mix thoroughly.

3. Let the sample stand at room temperature for 15 minutes, then centrifuge for one and one-half minutes.

4. Transfer 100 µl of the supernatant solution to a clean 400 µl polyethylene test tube. Stopper these tubes and let them stand to be analyzed together with the samples that have been incubated (Procedure, Paragraph 1).

When the prescribed period of incubation of the phosphatase tubes is complete (Procedure, Paragraph 1), proceed as follows:

5. Add 50 µl of 30 percent trichloroacetic acid to each of the phosphatase tubes. Mix thoroughly and let stand for 15 minutes at room temperature.

6. Centrifuge these tubes for one and one-half minutes and transfer 100 µl of the supernatant solution to clean 400 µl polyethylene test tubes.

Then to both the phosphatase tubes and to the tubes prepared for serum inorganic phosphorus analysis, continue as follows:

7. Add 20 µl of molybdic acid to the transferred supernatant solution and mix, then add 20 µl of ANSA and mix thoroughly. Let the tubes stand for 15 minutes at room temperature then read them against water at 650 mµ.

The method was developed and subsequent data were collected using the Spinco spectrocolorimeter, the upper wavelength limit of which is 650 mµ. Other instruments set at higher wavelengths may be used equally well.

C. Calculation

$$\frac{\text{Absorbance (A) of unknown - A of blank}}{\text{A of standard - A of blank}} \times 4 = \text{concentration of inorganic phosphorus (mg/100 ml)}$$

Phosphorus determined in alkaline phosphatase (incubated) tubes	-	inorganic phosphorus determined	=	alkaline phosphatase in units

Calculate the inorganic phosphorus in the tubes which have been incubated and those which have not in the same manner. A unit of phosphatase activity is defined as the amount of reaction that will release one mg of inorganic phosphorus per 100 ml of serum after one hour incubation at 37°C under the conditions of pH, buffer concentration, and type of substrate herein described.

RESULTS

A. Linearity of the Reaction

In order to demonstrate the linearity of the reaction, alkaline phosphatase was obtained as a dry powder from Nutrional Biochemical Corporation and dissolved in 0.9 percent sodium chloride solution. It was then diluted with the sodium chloride solution until the activity could be determined. Aliquots of this solution were then further diluted 1:2, 1:4, and 1:8. The phosphatase activity of each of these dilutions was then determined and expressed in units. The enzyme activity is plotted against the fractional dilution in Figure 1. A linear plot was obtained indicating that the catalytic release of phosphorus is directly related to the activity of the enzyme in the range possible with the procedure.

B. Comparison of Ultramicro and Macro Procedures

A comparison of the results obtained by a macro procedure (3) with those obtained by the ultramicro procedure using human serum are listed in Table 1. In none of the determinations did the macro and ultramicro results vary as much as one unit. The maximum

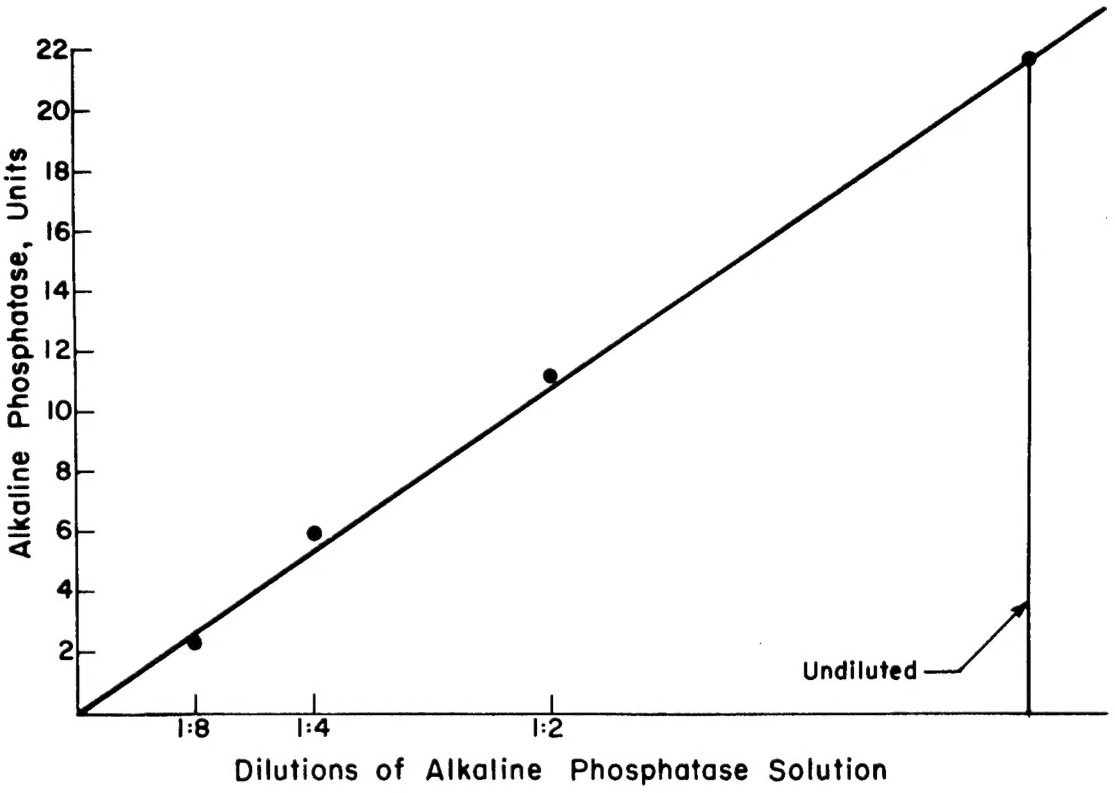

Fig. 1. Absorbance values which show linearity of the ultramicro alkaline phosphatase procedure described in the text.

TABLE 1

COMPARISON OF THE MACRO AND ULTRAMICRO PROCEDURES

The inorganic phosphorus and alkaline phosphatase were determined on the same sample by the macro and ultramicro procedures.

	Inorganic Phosphorus mg per 100 ml			Alkaline Phosphatase Units		
Sample	Macro	Ultramicro	Difference*	Macro	Ultramicro	Difference*
1	4.5	4.9	+0.4	10.3	9.4	-0.9
2	3.7	4.0	+0.3	2.1	2.1	0.0
3	3.8	3.7	-0.1	4.4	4.5	+0.1
4	4.2	3.9	-0.3	9.3	9.3	0.0
5	4.3	4.4	+0.1	8.1	7.8	-0.3
6	2.8	3.1	+0.3	3.0	2.9	-0.1
7	3.6	3.8	+0.2	4.2	4.0	-0.2
8	3.5	3.6	+0.1	3.1	3.0	-0.1
9	3.3	3.6	+0.3	3.9	3.9	0.0
10	2.8	2.9	-0.1	2.8	3.5	+0.7
11	3.7	4.0	+0.3	6.0	6.7	+0.7
12	4.5	4.4	-0.1	3.1	3.1	0.0
13	2.7	3.0	+0.3	3.9	3.6	-0.3
14	3.7	4.2	+0.5	7.5	7.0	-0.5
15	4.0	4.2	+0.2	5.4	4.6	-0.8

* The ultramicro result is compared to the macro result as reference.

variation was 0.9 unit but was 0.5 unit or more in only 5 of the 15 analyses. Variations in the determination of alkaline phosphatase by this method are influenced by variations in the determination of inorganic phosphorus originally present in the serum. The comparative results by the two procedures for inorganic phosphorus are also listed in Table 1. The ultramicro procedure compares favorably with the macro procedure for both inorganic phosphorus and alkaline phosphatase.

C. Standard Deviations

Thirty ultramicro alkaline phosphatase determinations were performed on the same sample of human serum. The mean value found was 3.4 units with a standard deviation of $\pm$ 0.05 mg of phosphorus per 100 ml of serum.

SUMMARY

An ultramicro procedure for the determination of alkaline phosphatase has been investigated. The method utilizes sodium β-glycerophosphate as the substrate at pH 9.3, and the phosphorus is determined by ultramicro analysis of inorganic phosphorus using 1-amino-2-naphthol-4-sulfonic acid as the reducing agent (10). Results with the method are compared to results obtained with the macro method from which the ultramicro method was derived.

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